FINAL (NO. 3) *REPORT ON*

CONTRACT NO DA -92-557-FEC-37654

INCLUSIVE DATES 8 September, 1964 *TO* 7 September, 1965

SUBJECT OF INVESTIGATION

PURIFICATION

OF

HISTAMINE SENSITIZING FACTOR

OF

BORDETELLA PERTUSSIS

RESPONSIBLE INVESTIGATOR

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Abstract

Purification of histamine sensitizing factor (HSF) of Bordetella pertussis was attempted from two sources : i.e. sonicate of the bacterial cells and the culture supernatant.

1. Subcellular fractions were prepared from the cells disintegrated by sonic oscillation. A fraction sedimentable at 105,000 x *g* for 90 minutes contained potent HSF activity as well as lethal toxicity. This fraction was mainly composed of ribosome particle.

2. Electrophoretic experiments and ultracentrifugal analysis of the ribosomal fraction digested with protease or ribonuclease indicated that HSF activity can not be refered to the ribosome particle itself.

3. HSF could be extracted from the ribosomal fraction with surface active agents such as sodium deoxycholate. Lethal toxicity was completely disappeared by this treatment.

4. Several methods for purification of HSF from the culture supernatant, such as density gradient electrophoresis, pH gradient elution and polyvinyl pyrrolidone treatment, were surveyed.

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CONTENTS

1. Introduction

Purification of histamine sensitizing factor (HSF) of Bordetella pertussis was undertaken by several workers (2, 3, 9, 10, 13), but no homogeneous preparation has so far been obtained. Our preparation purified from the culture supernatant of the microorganism (6, 7, 11) may also seem to be inhomogeneous. From our past experiences (11, 12), the general characteristics concerning HSF may be summarized as follows :

a. Easily diffusible from cells to medium during growth
b. Mainly found in "cell wall" and "ribosomal" fractions
c. Relatively heat labile
d. Inactivated by formaldehyde without losing the antigenicity.
e. Stable between pH 4.4 - 9.6, while labile at pH 1.8 or 12.
f. Relatively stable in frozen or lyophilized state for months
g. Irreversibly adsorbed on Seitz or glass filter
h. Precipitated by ammonium sulfate at 0.3 - 0.6 saturation
i. Adsorbed by DEAE cellulose (pH 7 or more), but not by CM cellulose (pH 6.1)
j. Precipitated at pH lower than 6.2 in low ionic strength medium.

In this periods of the Contract from 8 September, 1964 to 7 September, 1965, all the efforts have been made to obtain a homogeneous preparation of HSF from the cells and the culture supernatant. It was very difficult to attain complete isolation of HSF because of its slight solubility and of its polydispersion.

2. Materials and Methods.

a. Cultivation. Bordetella pertussis strain 18-323 (phase I) was used throughtout the experiments. The cells for fractionation were harvested from 72 hours culture on an agar medium (Table 1), and washed three times with the buffered saline (0.85 % NaCl, M/20 phosphate buffer pH 7.2 containing 5mM $MgSO_4$). For the culture supernatnt we used the K-medium as described in the preceding report (6).

b. Preparation of subcellular fractions. The washed cells were submitted to sonic oscillation (10 Kc) for 4 minutes under cooling circulation. The sonicate were fractinated by differential centrifugation as shown in Fig. 1. Each fraction is designated as 20-P fr. (intact cells, cell debris and cell walls), 105-P fr. (ribosomal fraction), 105-S fr. (supernate of ribosomal fraction), D-105-P fr. (debris of ribosomal fraction) and W-105-P fr. (washed ribosomal fraction). Further fractionations and treatments were done as specified in Results.

c. Biological assays. HSF activity and toxicity were

assayed using dd mice as described in the preceding reports(6, 7). The potencies were expressed by recording number of mice died per challonged instead of HSD_{50} and LD_{50} calculated by Behrens-Kaerber 's method, because the fifty doses were found to be not exact necessarily.

d. Chemical analyses. Nucleic acids and protein were determined by Schmidt & Thannhauser's method and Lowry's method respectively.

e. Ultracentrifugal analysis. Sedimentation coefficients of the ribosomal fraction were calculated from the data of ultracentrifugation with Hitachi Analytical Ultracentrifuge Model UCA-1.

3. Results

a. HSF activity and lethal toxicity of the subcellular fractions. Preliminary attempts reported in the Final Report (No.2) showed that most of the HSF activity in the protoplasm was found in the fraction sedimentable by centrifugation at 105,000 x g for 90 minutes, which is composed mainly of ribosomes (5, 7, 8). In order to re-examine the result, localization of HSF in the subcellular fractions of pertussis cells was investigated by the method indicated in Fig. 1. Sonication of the intact cells resulted in considerable increase in HSF as well as lethal toxicity for mice. HSF activities of 105-P, W-105-P and 105-S fractions were approximately as same as that of the intact cells (Table 2). The HSF activity of the ribosomal fraction did not change following washing with buffered saline. As shown in Table 3, the RNA content of W-105-P fr. increased up to 30 % from about 20 % of 105-P fr. The electron micrography of W-105-P fr. revealed the presence of relatively homogeneous particles of about 250 Å in diameters (Plate I). From these results, it may be suggested that HSF and lethal toxic activities of 105-P fr. are closely associated with the ribosomal fraction.

b. Extraction of HSF from the ribosomal fraction. In order to solubilize HSF from the ribosomal fraction, the following surface active agents were tested.

(1) Sodium dodecyl sulfate (SDS)
(2) Sodium deoxycholate (SDC)
(3) Tween 20
(4) Alkyl trimethyl ammonium chloride

The agents were added to 105-P fr. and incubated at 37° C for 15 minutes. Judging from the reduction in turbidity of the suspension of 105-P fr., SDS and SDC only out of the four agents tested were effective for the dissolution of the suspension. The final concentrations of SDS and SDC to attain 50 % reduction in the turbidity were about 0.05 and 0.1 % respectively. SDS as well as SDC were found to be able to extract HSF from 105-P fraction, whereas the lethal toxicity was not found in the extracts. Accordingly SDC was employed in most case. The details of the extraction procedure are shown in Fig. 2. The extract, i.e. supernatant of centrifugation at 105,000 x g for 90 minutes, was dialysed against running tap water for 48 hours in order to remove the surface ac-

tive agent. The slightly turbid, dialysed extract was spun at 105,000 x g for 60 minutes. and supernatant (105-PES. soluble fraction) and residue (105-PEP) were obtained. As shown in Table 4, HSF activity of 105-PE and 105-PR fractions goes share and share alike. Most of HSF activity in 105-PE fraction was found in the precipitate fraction (105-PEP) occurring during dialysis (Table 5). Distribution of RNA and protein among the fractions are given in Table 6. Most of RNA in 105-PE fraction remained in 105-PES fraction, leaving only 7.1 % in 105-PEP fraction, while about one third of the protein was found in 105-PEP fraction. In this connection it may be of interest that HSF activity of the preparation obtained from the culture supernatant was also found in the precipitate formed during prolonged dialysis (11).

c. Enzymatic treatment of 105-P fraction. The suspension of 105-P fraction was treated with Nagarse (unspecific protease derived from Bacillus subtilis) and pancreatic ribonuclease (RNase) as shown in Fig. 3. After incubation at 37° C for 30 minutes with or without the enzymes the reaction mixtures were spun at 105,000 x g for 90 minutes, and the precipitate and the supernatant fractions were assayed for protein and RNA contents (Table 7). The HSF activities of the precipitate fractions following the enzyme treatments are given in Table 8. Neither Nagarse nor RNase gave any profound change in HSF activity of the fractions, while excess release of protein or RNA by the treatment was observed as seen in Table 7.

Washed ribosomal fraction (W-105-P fr.) suspended in phosphate buffer containing 5 mM $MgSO_4$ was submitted to ultracentrifugal analysis. Schlieren pattern of W-105-P fr. (Plate II-A) showed three peaks (25, 50 and 68 S respectvely) in which the 68 S peak was predominant one, in agreement with the results obtained for ribosomes of many bacterial species. Following the treatments with RNase (Plate II-B) and with Nagarse (Plate II-C), peaks having 68 S and 50 S were belittled or disappeared completely, while 25 S peak remained unchanged. As mentioned above, the enzymatic treatments did not result in any profound loss of HSF activity. For this reason, it seems likely that the active principle of HSF may probably be associated to the 25 S particles or to other minor components sedimentable at 105,000 x g for 90 minutes. Attempts to separate each particles by sucrose-gradient centrifugation are now being carried out.

d. Zone electrophoresis of 105-P fraction. It is desirable to determine whether the active principle of HSF found in 105-P fraction can be refered to ribosome particle itself or not. To examine this alternative possibilities, zone electrophoresis of 105-P fraction on a starch block was carried out. About 500 mg of lyophilized 105-P fraction was suspended in 2 ml of M/20 phosphate buffer, pH 7.02 containing 5 mM $MgSO_4$ were placed in a starch block (42 cm long, 4.9 cm width and 1.6 cm depth). Electrophoretic run was carried out in a cold room (about 4° C) as specified in Fig. 4. Judging from the protein and RNA content and ultraviolet absorption curve (Fig. 5), fraction 5, which is rich

in RNA, was not compatible with any peaks of HSF and lethal toxicity . HSF and lethal toxicity spread through quite a broad zone suggesting polydisperse nature of the both activities. To verify that ribosome itself is not identical with the active principle of HSF, RNA rich fraction 5 was collected and re-electrophoresis was carried out as shown in Fig. 6. RNA rich peak was again found about fraction 5' migrated toward anode, however, HSF activity was found arround fraction 2 - 3 which contained very minute amount of RNA(Fig. 6 and 7). A peak of lethal toxicity exsited in fraction 3. These data demonstrate that HSF as well as lethal activities can not be refer to ribosome particle itself.

e. On the possibility of adsorption of HSF on the ribosomal fraction. In consideration of the possibility that HSF happened to be adsorbed on the ribosome particles, the following experiment was carried out. The ribosome fraction was prepared from phase IV Bordetella pertussis strain Karasumori, which is free from HSF, by the method described above (see Fig. 1). To this ribosomal fraction, culture supernatant of phase I Bordetella pertussis (strain 18-323) which contained potent HSF was added. Following incubation at 37° C for 30 minutes and then keeping at 4° C overnight (Fig. 8), the mixture was centrifuged at 105,000 x g for 90 minutes. The precipitate was tested for HSF activity (Table 9). It is clearly demonstrated that the possibility of adsorption of HSF on the ribosomal fraction derived from phase IV (HSF free) cells is negligible. This result does not necessarily exclude the adsorption of HSF on phase I ribosome, but the HSF activity found in 105 -P fraction from phase I cells may be refered to some component other than ribosome itself which concomitantly sedimented in the fraction.

f. Purification of HSF from the culture supernatant.(4)
Several attempts to purify HSF from the culture supernatant of Bordetella pertussis phase I strain 18-323 were done. Partially purified preparation (P1 fraction as reported in the preceding reports) was used as a starting material.

(1) Gel filtration of P1 fraction. P1 fraction suspended in a medium (Na_2HPO_4, M/15, + 0.5 M NaCl, pH about 9) was passed through a Sephadex G-200 column and eluted with the same medium. HSF was eluted in fractions No. 3 - 8 (Fig. 9). Elution with lower pH and lower ionic strength (M/50 phosphate buffer, pH 8.0 + 0.2 M NaCl) gave several peaks, and HSF was found in peaks A and B (Fig. 10). It seems that HSF has a considerable large molecular weight.

(2) pH gradient elution of HSF from P1 fraction. P1 fraction dissolved in phosphate buffer (pH 8.33, M/5) was centrifuged at 10,000 x g for 15 minutes, and the supernatant was dialysed against cold deionized water for 48 hours. Massive precipitate was formed upon thawing the dialysed solution frozen in a deep freezer (about -25° C). No such precipitate was observed in the case of undialysed supernatant. The freeze-thawing procedure repeated twice as shown in Fig. 11. Most of HSF activity was

found in the fraction FR1 and FR2. The combined precipitate fraction was lyophilized and charged on a short column of Hyflo-supercel and eluted with buffers as specified in Fig. 12. HSF activity was found in fraction D and E. This method seems to be promising for further purification of soluble HSF.

(3) Density gradient electrophoresis. Electrophoresis in sucrose density gradient column was carried out using an apparatus developed in our laboratory (Fig. 13). Lyophilized sample was suspended in Tris-HCl buffer (pH 8.6, μ = 0.05) containing 2 % sucrose, and mounted on the surface of sucrose density layer (20 - 5 %). Results with P1 are given in Fig. 14A. There were three HSF peaks. Fraction 12 - 16 were submitted to reelectrophoresis and gave one peak (Fig. 14B), but HSF activity was lost. This result indicate polydispersed nature of HSF.

(4) Treatment with polyvinyl pyrrolidone. Polyvinyl pyrrolidone is used in fractionation of serum lipoprotein. P1 was suspended in phosphate buffer (M/10, pH 7.9) containing 10 % of NaCl, and polyvinyl pyrrolidone solution was added to the suspension to give various final concentrations. The precipitate was removed by centrifugation as shown in Fig. 15. The supernatants having potent HSF activity and low protein content were obtained (Table 10). Each fractions showed two precipitation lines against antiserum immunized with sonicate of the homologous cells in Ouchterlony plate (Fig. 16). Though this method may be promising for enhancing specific activity of HSF, further investigation should be necessary.

4. Discussion and Conclusion.

Most of workers engaging in purification of HSF as well as mouse protective antigen of Bordetella pertussis are trying to extract from the cells (2, 3, 9, 10, 13). In the present report we described several attempts to purify HSF from the bacterial cells and the culture supernatant. Although HSF activity was found in a fraction sedimentable at 105,000 x g for 90 minutes, which contained ribosomes (5, 8, 12), it is concluded from the electrophoretic and ultracentrifugal experiments that the HSF activity can not be refered to ribosome particle itself. Recently Sato and Nagase (14) have succeeded in obtaining very potent mouse protective antigen from the ribosomal fraction of Bordetella pertussis. This ribosome fraction showed protective potency as well as HSF activity, in agreement with our results, but further separation of the protective antigen by sucrose density gradient centrifugation demonstrated that their protective antigen having a sedimentation coefficient of about 21 S, showed no HSF activity. It may be very difficult to draw any conclusion on the relationship between the protective antigen and HSF until molucular homogeneity of both entities well be established.

Slight solubility of HSF gives an obstacle to the purification. Extraction of HSF in a soluble, homogeneous state may be desirable. Surface active agents such as SDC or SDS can be employed for the solubilization of HSF from 105-P fraction as well as from rather insoluble preparation. SDC has been used for extraction of cell-

ullar components from Bordetella pertussis cells by several workers (1. 14). Purification from the culture supernatant is advantageous for large scale preparation. Several methods of purification were surveyed in this period. Judging from the electrophoretic data, P1 derivel from the culture supernatant seems to be polydisperse. Recently, Pieroni et al. (13) reported about eight-fold purification of HSF from mechanically disintegrated cells. They also suggested polydisperse nature of HSF as well as the protective antigen, and tentatively proposed possible identity of HSF with the protective antigen. It is of interest that HSF activity goes to the precipitate formed upon thawing of frozen sample. Polyvinyl pyrrolidone treatment is also useful for enhancing the specific activity because most of inactive proteinēous contaminant were removed. Further purification of HSF will be continued.

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Appendix

Table 1. Composition of the semi-synthetic medium

Casaminoacid (Difco, technical)	10.0 g
KH_2PO_4	1.0 g
NaCl	5.0 g
$MgCl_2 \cdot 6H_2O$	0.4 g
Cystein hydrochloride	0.1 g
Nicotinic acid	10 mg
Yeast dialysate	50 ml
Monosodium glutamate	2.5 g
Haemin*	4 mg
Nucleo**	50 mg
Spermine tetrahydrochloride	1 mg
Agar (purified)	20 g
Charcoal (Norit)	1.0 g
Water added to	1,000 ml
pH was adjusted to 7.2	

*Haematin hydrochloride, Fe: 8.11 %

**Alkaline hydrolysate of yeast RNA (Nippon Zoki Co.)

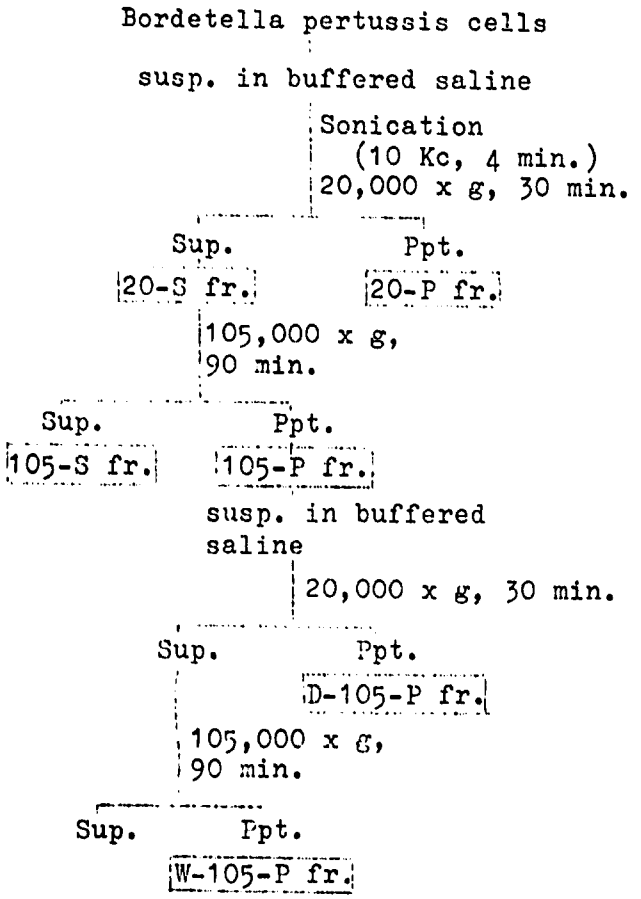


Fig. 1. Preparation of subcellular fractions from Bordetella pertussis cells

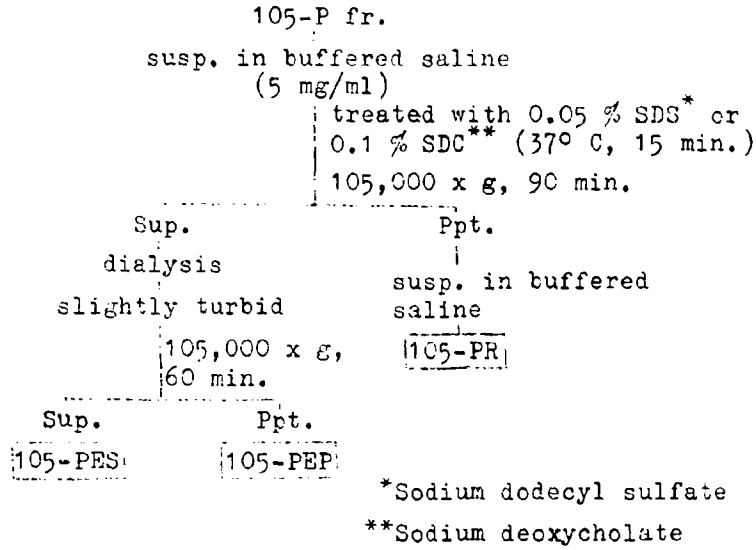


Fig. 2. Extraction of HSF from 105-P fr. with surface active agents

Table 2. HSF and toxicity of subcellular fractions

Fraction	Dose per mouse	HSF activity (Died/Tested)	Toxicity (Died/Tested)
Intact cells	1/3 mg	6/10	
"	1/9	4/10	7/10
"	1/27	2/10	0/10
"	1/81		0/10
105-P fr.	1/3 mg	6/10	
"	1/9	4/10	10/10
"	1/27	2/10	10/10
"	1/81		1/10
W-105-P fr.	1/3 mg	7/10	
"	1/9	3/ 9	10/10
"	1/27	1/10	7/10
"	1/81		1/10
D-105-P fr.	1/3 mg	3/10	
"	1/9	2/10	4/10
"	1/27	2/10	2/10
"	1/81		0/10
105-S fr.	1/3 mg	5/10	
"	1/9	4/10	10/10
"	1/27	1/10	10/10
"	1/81		9/10

Table 3. Protein and RNA content of subcellular fractions

Fraction	RNA	Protein (as BSA*)	RNA/Protein
105-P fr.	21.1 %	63.2 %	0.34
105-S fr.	11.2	68.3	0.16
W-105-P fr.	30.0	56.0	0.54
D-105-P fr.	16.8	51.9	0.32

*Bovine serum albumin

Table 4. Biological activities of 105-PE fr.

Extracted with	Dose per mouse	HSF activity (Died/Tested)	Toxicity (Died/Tested)
SDS*	(1.0 mg)***	7/7	0/8
"	(1/3 mg)	0/6	0/8
"	(1/9 mg)	2/8	0/8
SDC**	(1.0 mg)	4/4	0/8
"	(1/3 mg)	6/8	0/8
"	(1/9 mg)	5/8	0/8
105-P fr.	1.0 mg	8/8	8/8
"	1/3 mg	8/8	8/8
"	1/9 mg	5/8	7/8

*Sodium dodecyl sulfate
**Sodium deoxycholate
*** ( ) : Equivalent dose to 105-P fr.

Table 5. HSF activity of 105-P fr. and other fractions

Fraction	Dose per mouse	HSF activity (Died/Tested)
105-P fr.	1/3 mg	9/10
"	1/9	4/10
"	1/27	2/10
105-PE fr.	1/3 mg	8/10
"	1/9	2/ 9
"	1/27	0/10
105-PEP fr.	1/3 mg	10/10
"	1/9	5/ 9
"	1/27	2/10
105-PES fr.	1/3 mg	3/ 9
"	1/9	2/ 9
"	1/27	0/10
105-PR fr.	1/3 mg	8/10
"	1/9	3/10
"	1/27	0/10

Table 6. Distribution of RNA and protein into 105-P and other fractions

	RNA		Protein		RNA/Pr.
105-P	(100.0 %)		(100.0 %)		0.36
105-PR	55.0 %		61.1 %		0.06
105-PE	10.8 %	(100.0 %)	35.6 %	(100.0 %)	0.55
105-PEP		7.1 %		35.2 %	0.20
105-PES		92.0 %		64.0 %	0.76

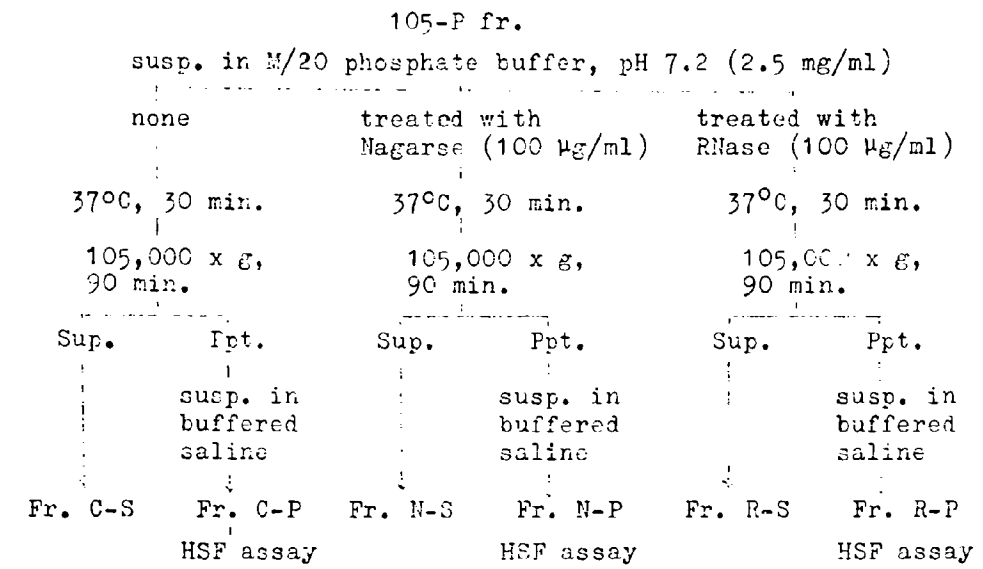


Fig. 3. Treatment of 105-P fraction by enzymes

Table 7. Protein and RNA content of 105-P fraction after treatment by enzymes

Fraction*	R N A	Protein
Fraction C-S	3,840 μg (58.0 %)	4,560 μg (27.6 %)
Fraction C-P	2,820 μg (42.0 %)	12,000 μg (72.4 %)
Fraction N-S	3,820 μg (56.0 %)	10,680 μg (63.9 %)
Fraction N-P	3,000 μg (44.0 %)	6,000 μg (36.1 %)
Fraction R-S	4,950 μg (72.0 %)	5,160 μg (30.1 %)
Fraction R-P	1,920 μg (28.0 %)	12,000 μg (69.9 %)

*Refer to Fig. 3.

Table 8. HSF activity of enzyme treated 105-P fraction

Treated by	Dose per mouse*	HSF activity (Died/Tested)
Nagarse	(1.0 mg)	6/8
"	(1/3)	8/9
"	(1/9)	0/9
"	(1/27)	1/9
RNase	(1/3 mg)	7/9
"	(1/9)	4/9
"	(1/27)	1/9
None	1/3 mg	8/9
"	1/9	3/9
"	1/27	1/9

* ( ) : Equivalent dose to 105-P fr.

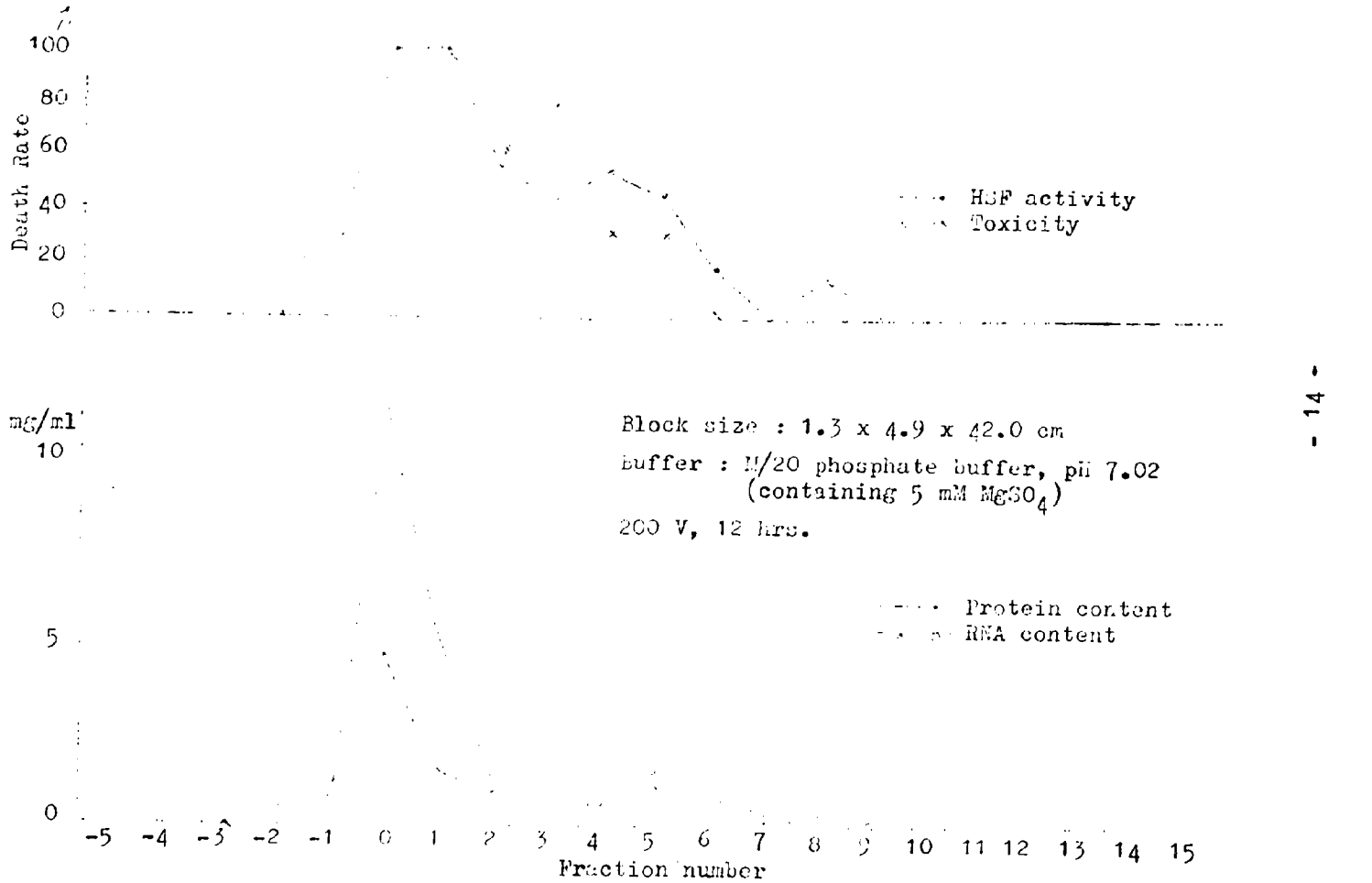


Fig. 4. Zone electrophoresis of 105-P fr. on starch block

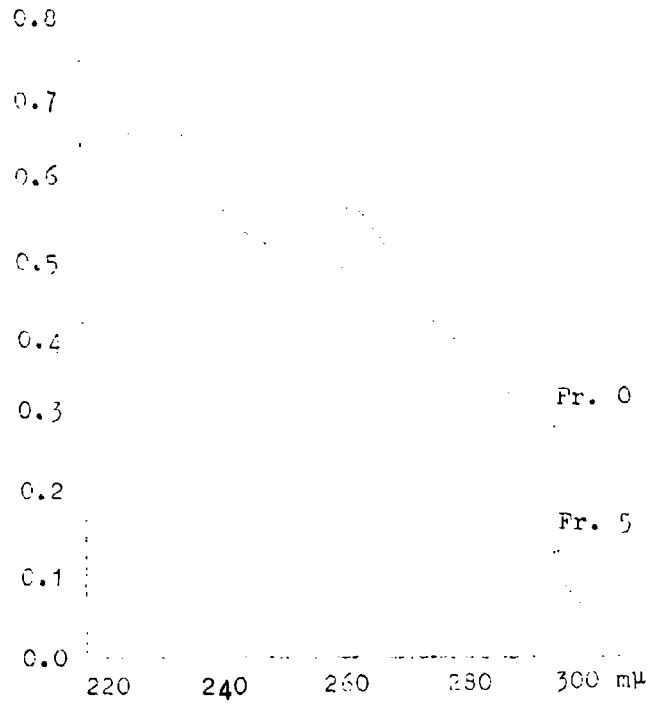


Fig. 5. Ultraviolet absorption spectrum of fr. 0* and fr. 5*

*Refer to Fig. 4.

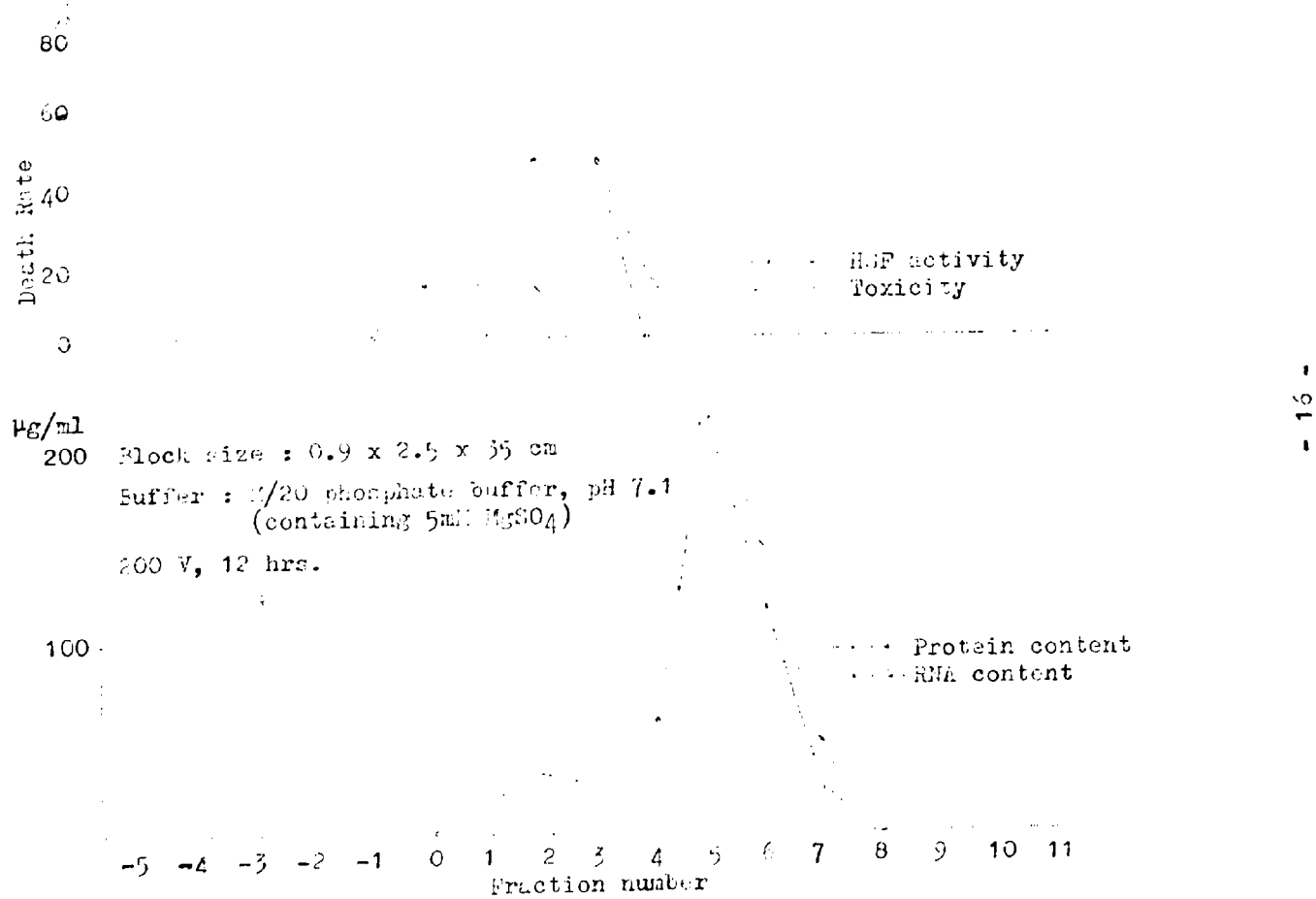


Fig. 6. Re-zone electrophoresis of fr. 5 obtained from first zone electrophoresis

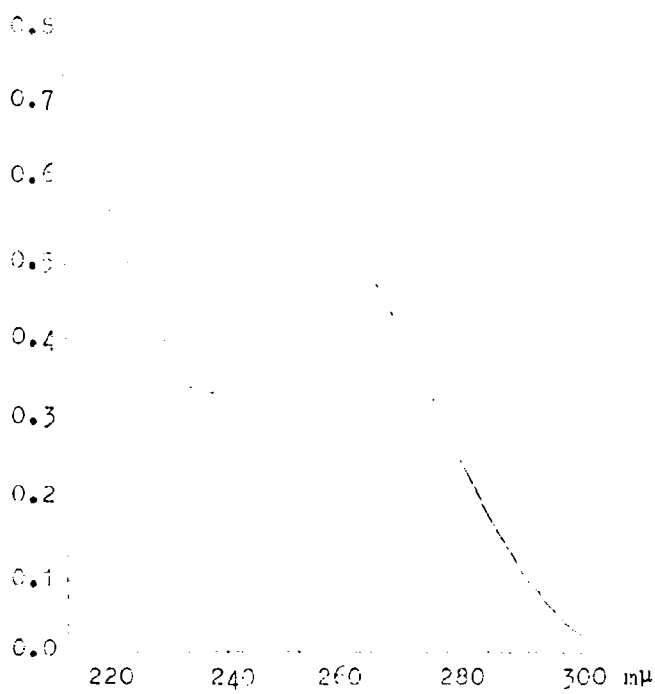
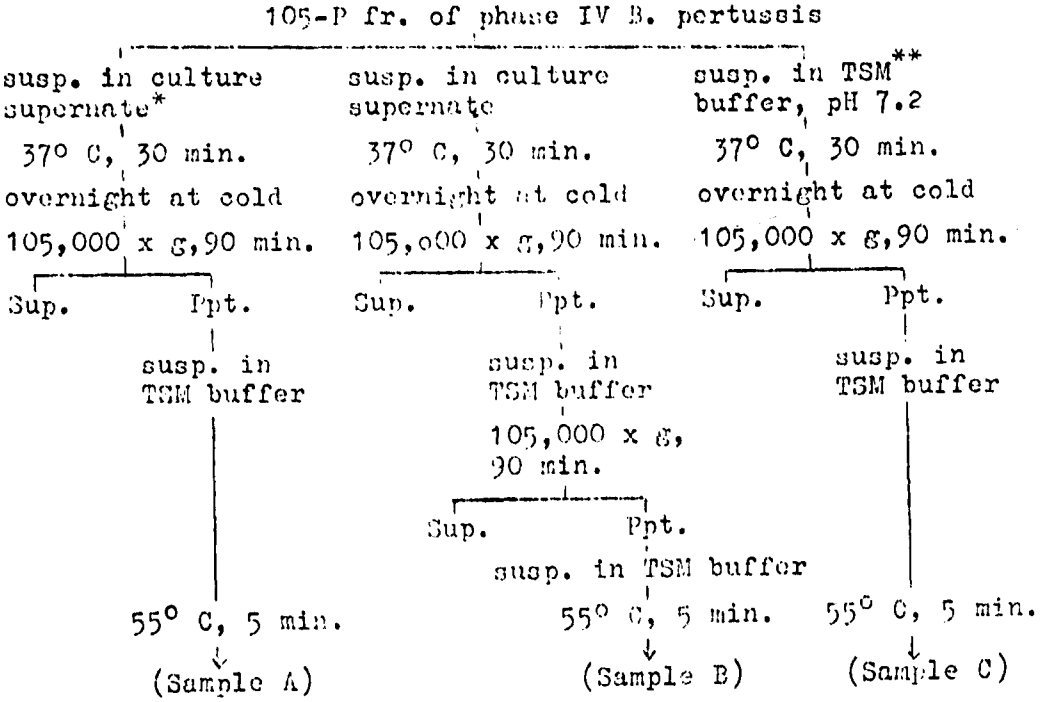


Fig. 7. Ultraviolet absorption spectrum of fr. 5*

*Refer to Fig. 6.



*Obtained from 72 hours culture of B. pertussis phase I. strain 18-323 by centrifugation at 2,000 rpm for 30 min.

**Tris (0.01M)-succinic acid (0.004M)-magnesium acetate (5mM).

Fig. 8. Adsorption of HSF in the culture supernate on the 105-P fr. derived from cells of phase IV B. pertussis (strain Karasumori)

Table 9. HSF activity of 105-P fr. of phase IV B. pertussis treated by culture supernate of phase I B. pertussis

Sample*	Dilution factor	HSF activity (Died/tested)
Culture supernate	1 x	8/8
"	5 x	7/8
Sample A	1 x	0/8
"	5 x	0/8
Sample B	1 x	0/8
"	5 x	0/8
Sample C	1 x	0/8
"	5 x	0/8

*Refer to Fig. 8.

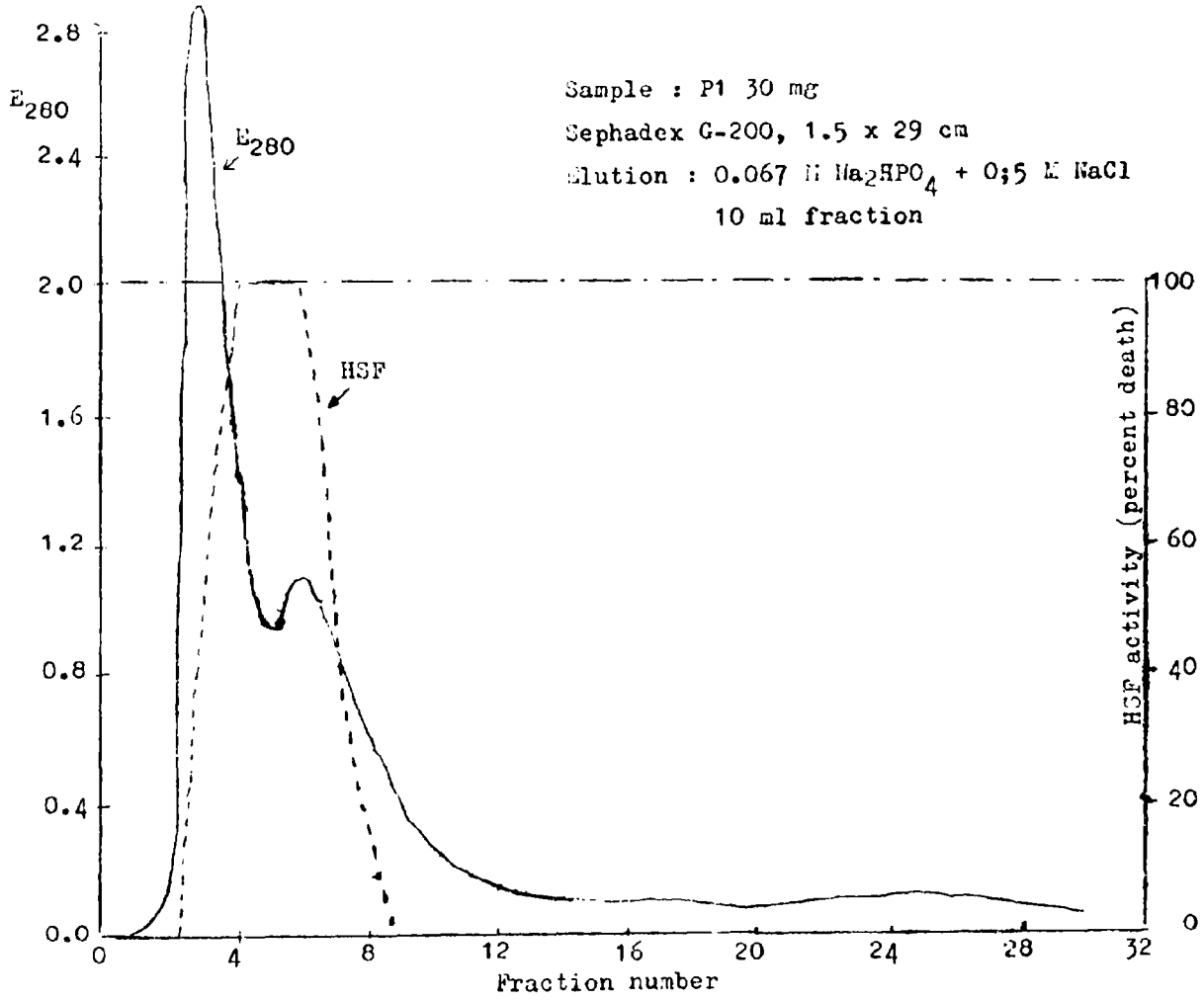


Fig. 9. Gel filtration of P 1

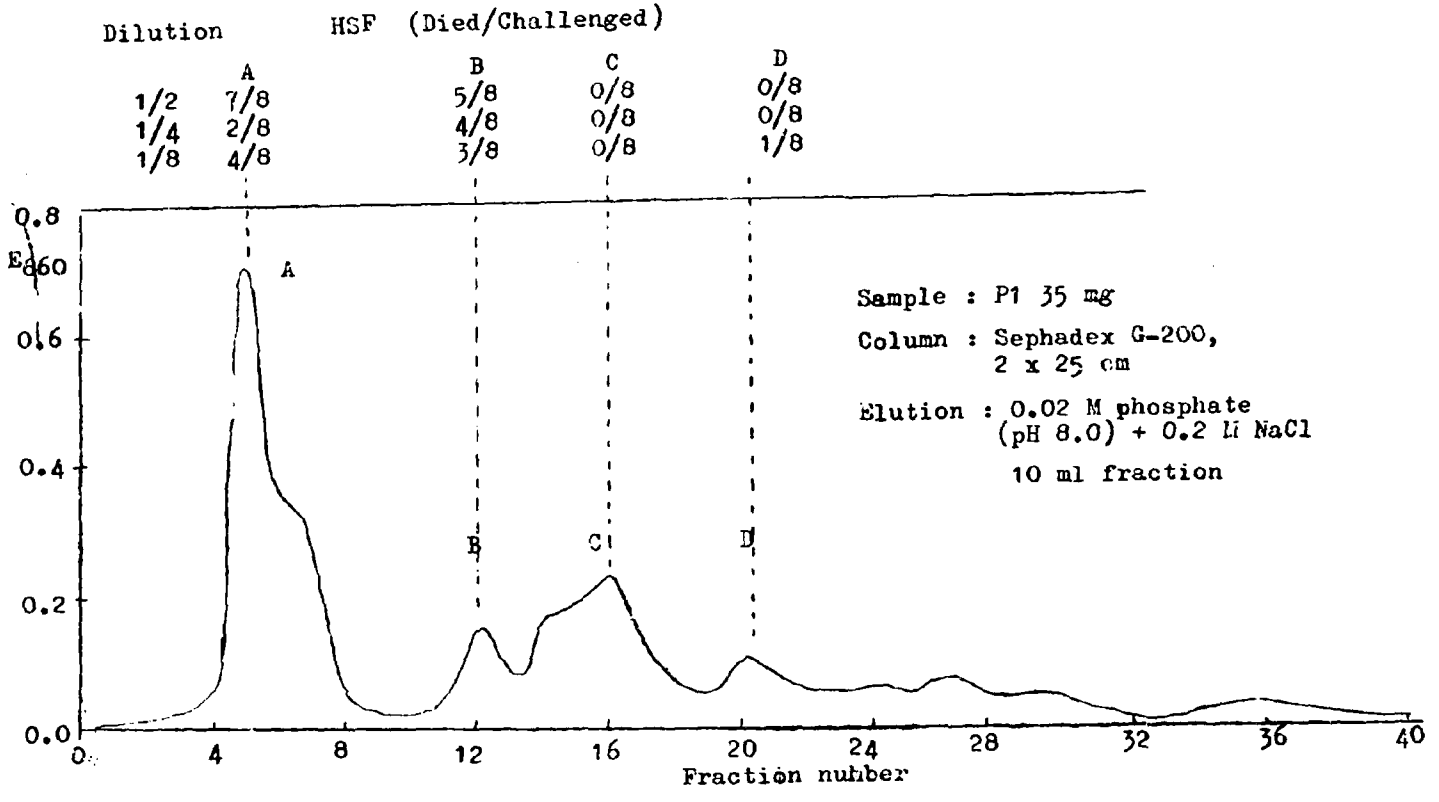


Fig. 10. Gel filtration of P1

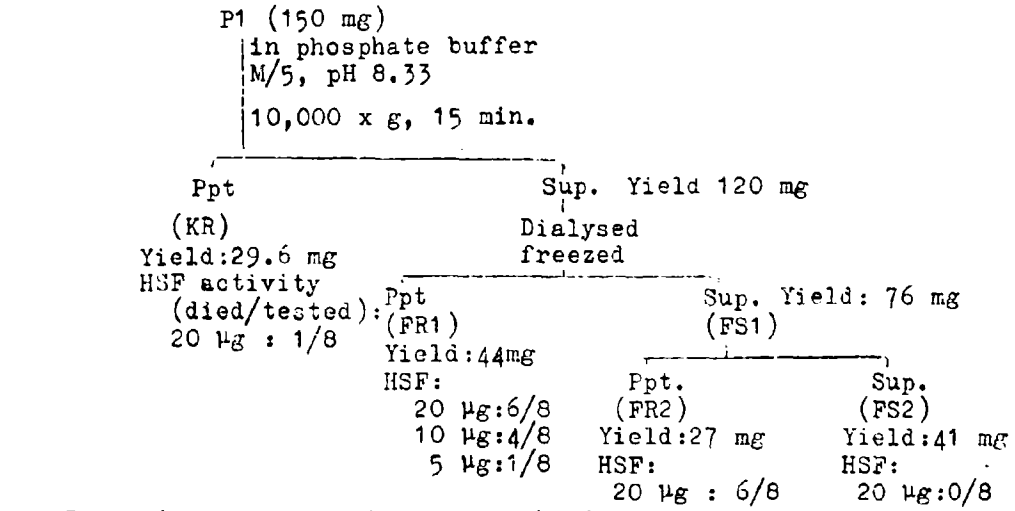


Fig. 11 Separation by freeze-thawing.

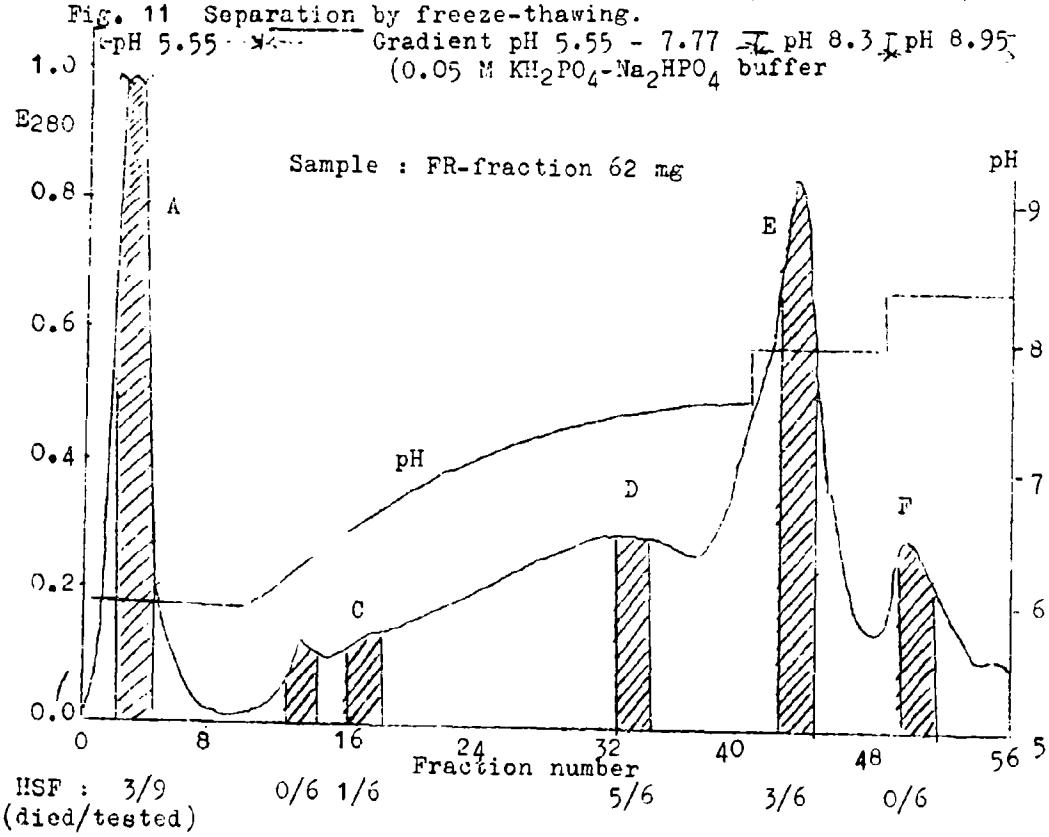


Fig. 12. pH gradient elution of FR fraction.

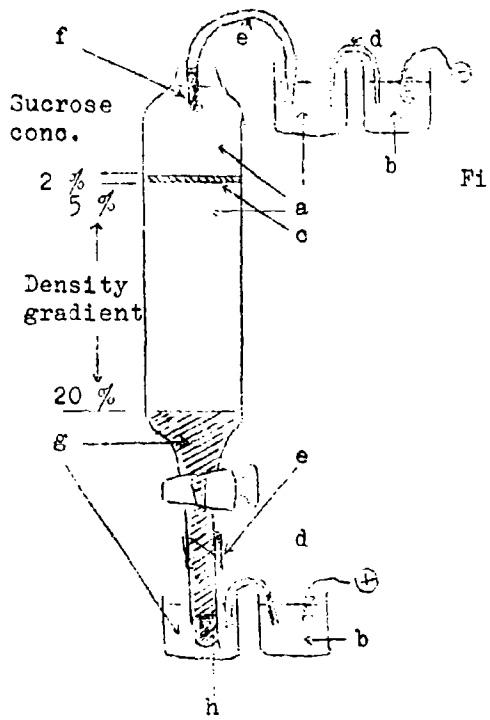
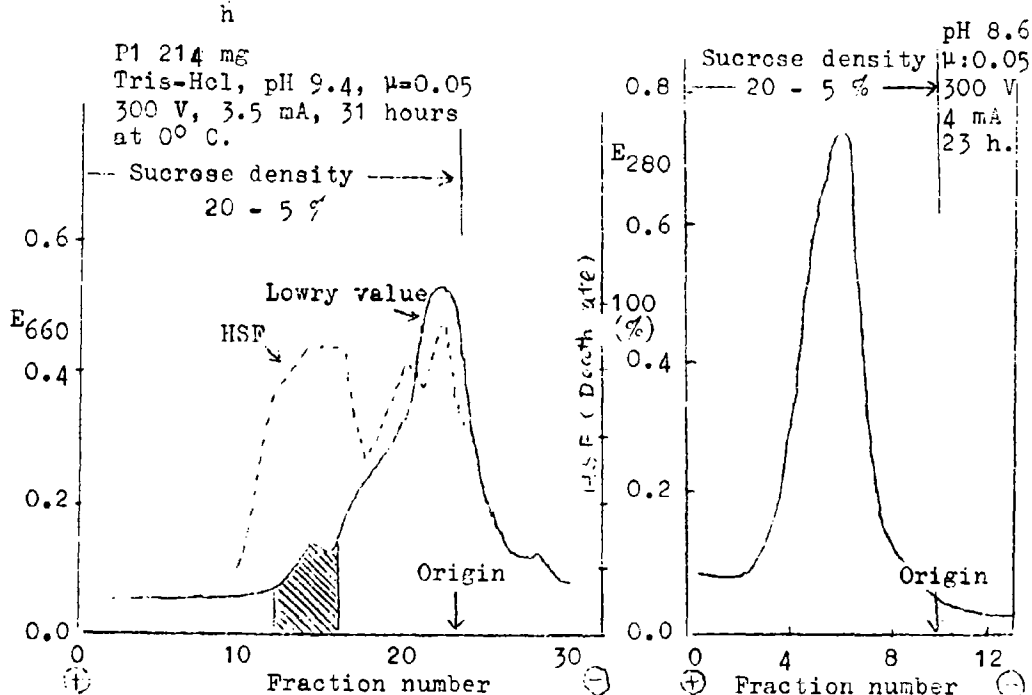


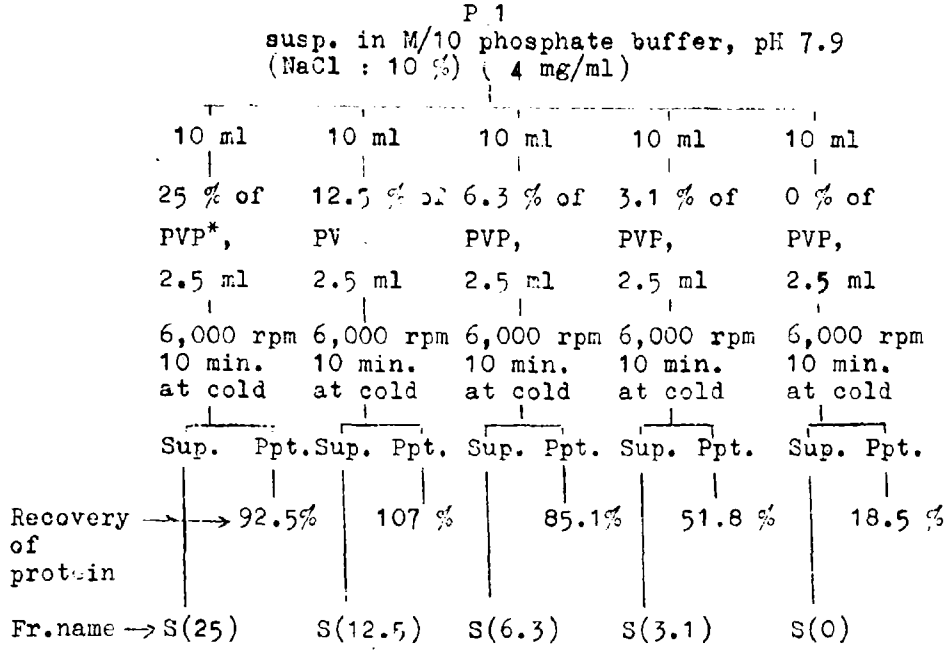
Fig. 13. An apparatus for density gradient electrophoresis

a. Tris-HCl buffer
b. 5 % KCl sol.
c. Sample
d. Agar bridge
e. Vinyl tube
f. Buffered agar bridge
g. Tris-HCl buffer (containing 20 % sucrose)
h. Buffered agar bridge (containing 20 % sucrose)



A. First electrophoresis B. Reelectrophoresis

Fig. 14. Density gradient electrophoresis of P1



*Polyvinyl pyrrolidone.

Fig. 15. Treatment of P1 with polyvinyl pyrrolidone

Table 10. HSF activity of polyvinyl pyrrolidone treated P1

Fraction*	Dilution factor	HSF activity (Died/Tested)
S(25)	1 x	8/8
		5/8
S(12.5)	1 x	6/7
	5 x	7/8
S(6.3)	1 x	6/6
	5 x	8/8
S(3.1)	1 x	8/8
	5 x	5/8
S(0)	1 x	4/7
	5 x	4/8
PVP (5 %)		0/8

*Refer to Fig. 15.

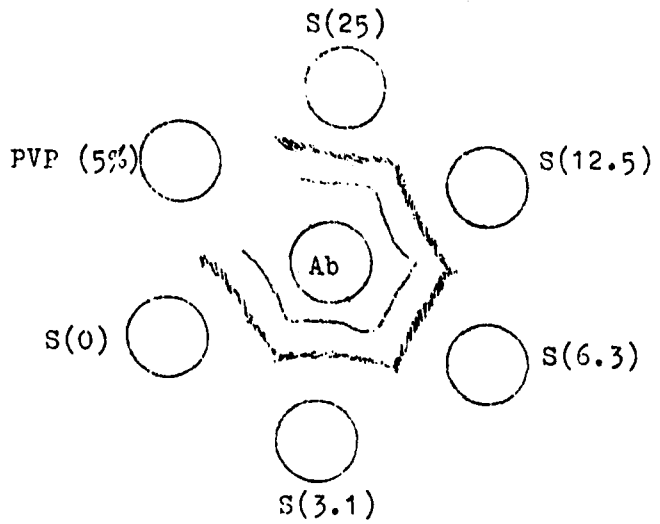


Fig. 16. Gel diffusion precipitation test (Ouchterlony)

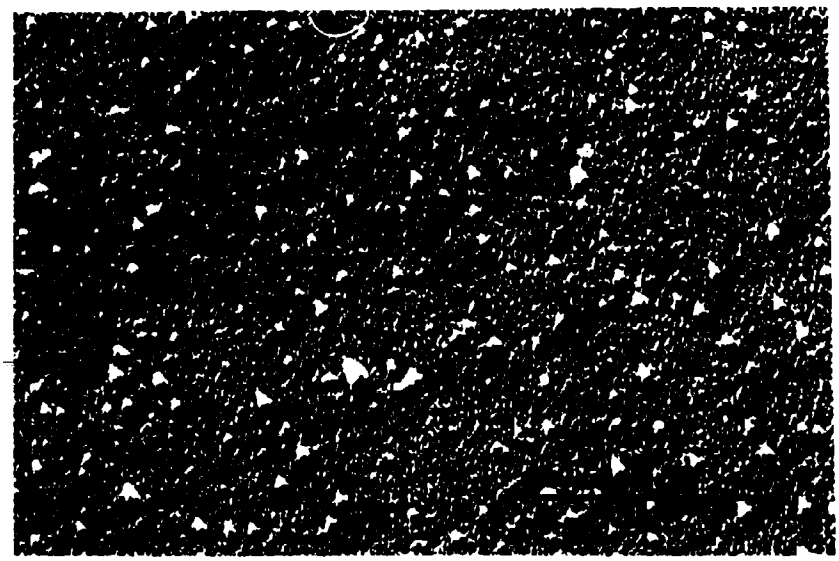


Plate I. Electron micrograph of W-105-P fr.
(shadowed by Pt-Pd)

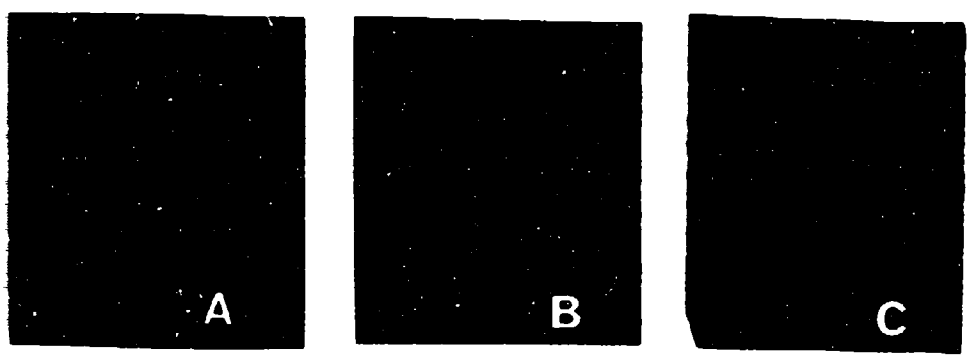


Plate II. Schlieren patterns of W-105-P fraction centrifuged at 40,370 rpm. The pictures were taken 12 minutes after maximum speed was attained.
A : Control W-105-P fraction.
B : RNase (20 μg/ml) treated W-105-P fr.
C : Nagarse (20 μg/ml) treated W-105-P fr.

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4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Final Report, 8 September 1964 - 7 September 1965

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13. ABSTRACT

Purification of histamine sensitizing factor (HSF) of Bordetella pertussis was attempted from two sources: i. e. sonicate of the bacterial cells and the culture supernatant.

1. Subcellular fractions were prepared from the cells disintegrated by sonic oscillation. A fraction sedimentable at 105,000 x g for 90 minutes contained potent HSF activity as well as lethal toxicity. This fraction was mainly composed of ribosome particle.

2. Electrophoretic experiments and ultracentrifugal analysis of the ribosomal fraction digested with protease or ribonuclease indicated that HSF activity can not be referred to the ribosome particle itself.

3. HSF could be extracted from the ribosomal fraction with surface active agents such as sodium deoxycholate. Lethal toxicity was completely disappeared by this treatment.

4. Several methods for purification of HSF from the culture supernatant, such as density gradient electrophoresis, pH gradient elution and polyvinyl pyrrolidone treatment, were surveyed. (Author)

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Histamine						
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Extraction						
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